16. The Denaturation of Edestin by Acid: T. B. Osborne's Edestan

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Certain denaturation products of the seed globulins, edestin and excelsin, have been studied by X-ray diffraction analysis [Astbury *et al.* 1935]. The fundamental observation emerging from this research was that a corpuscular protein could be denatured and manipulated to give a molecular pattern resembling that of β-keratin. We were tempted by this revelation to depart from the classical definition of protein denaturation, and to designate the β-keratin structure, or some approximation to it, as representing the final state of denaturation processes. At the same time, it was apparent that intermediate stages of protein degradation could be distinguished. Diffraction photographs of large air-dried excelsin crystals indicated a partial transition from the crystalline globular arrangement of the protein molecules to a more fibrous state. It seemed desirable at this stage to investigate in some detail the conditions governing the formation of these intermediate denaturation products, derived on the one hand from a molecule of precise and perhaps unique configuration, and giving rise by suitable treatment to a well-defined fibrous configuration.

At the beginning of the century, T. B. Osborne [1901; 1902, 1, 2] became interested in the formation of protein derivatives which he called *proteans*, and he gave particular attention to edest*an*, the protean derivative of hemp-seed globulin. The proteans were defined as the salt-insoluble globulin derivatives produced in small amounts during crystallization and preparative processes, and especially by the action of the most dilute acids. Osborne's careful experiments on the formation of edestan, hampered as they were by the meagre physico-chemical data existing at that time, must be considered as classical contributions which have not received the recognition they deserve. From our present standpoint, his most important observations were: (1) that the acid salt of the protein was the precursor of the denatured product; (2) that the amount of denatured protein formed in a given time depended upon the acidity of the solution; (3) that the transformation could proceed at a measureable rate. The denaturation of proteins by acids is a very general phenomenon. Ultracentrifugal studies in Svedberg's laboratory and elsewhere have shown that for each protein there is often a characteristic *p*H range outside the limits of which there may be irreversible denaturation changes, sometimes accompanied by the breakdown of the molecule into smaller components. Since the process is not peculiar to the plant proteins, and since the means of denaturation is one of many, it is now unnecessary to maintain Osborne's specific nomenclature in the case of acid-denatured globulins, except perhaps in the case of edestan, the classical and most investigated derivative of this type of denaturation change.

In the present study, several aspects of the breakdown of edestin to edestan have been investigated. Kinetical experiments have been designed to measure the effect of *p*H, protein concentration and temperature, and in addition, the denaturation mechanism has been studied by ultracentrifugal and X-ray analysis. Some chemical manifestations of the change, e.g. the liberation of SH groups, have also been followed. Wherever possible, analogies have been drawn between the formation of edestan and of the insoluble products formed during manipulation of protein-containing tissue extracts [Bailey, 1939].

It is important at the outset to stress that neither edestin nor the majority of other plant proteins can certainly be considered as completely homogeneous with respect both to particle weight and to electrical charge. This point is of primary importance in the consideration of the kinetical experiments. In most cases homogeneity has been established in the ultracentrifuge but has not been supplemented by electrophoretic or solubility experiments. That edestin is monodisperse in the ultracentrifuge was established by Svedberg & Stamm [1929] and confirmed by Philpot [present communication]. Electrophoretic experiments are rendered almost impossible by the extreme insolubility of edestin at low temperatures and low ionic strengths. The solubility of a 'standard' edestin preparation however was studied by the author in the laboratory of Dr E. J. Cohn of the Harvard Medical School, and was found to be greatly dependent upon the solvent-solute ratio, both in polar and dipolar solvents. By fractionation it was possible to obtain products differing in respect to gross solubility behaviour, but all attempts to obtain a fraction obeying the ideal solubility laws met with complete failure. This does not necessarily indicate that edestin is not homogeneous. In addition to the more generalized hypotheses advanced by Sørensen [1930; 1933], Bonot [1934; 1937] and Brønsted [1938], Steinhardt [1938, 1; 1939] has emphasized that the equilibrium between non-protein and protein components must be considered, and may exist independently of the dissolved phase. Such 'impurities' are known to occur in the case of pepsin and are shown here to be present in edestin. Yet it must be admitted that ideal solubility behaviour has often been achieved by removal of a protein rather than a non-protein impurity. The fractionation of horse-serum albumin by McMeekin [1939] is an excellent example. A simple phase-rule solubility, however, has generally been demonstrated only in the case of proteins with particle weights below 70,000. For large molecules like those of edestin, with a particle weight above 300,000, we may find that the laws governing the solubility of colloidal bodies hold more nearly, e.g. the Ostwald-Buzágh 'Bodenkörperregel'. No attempt has been made in subsequent experiments to derive conclusions which depend for their validity upon an assumed homogeneity both of particle weight and electrical charge.

Experimental

(1) *Preparation of edestin*

1 kg. of freshly ground hemp seed was extracted at 50° with 2 l. of 5 % NaCl for 1 hr. The extract was squeezed through calico and filtered hot through a 2 in. layer of filter pulp. The clear filtrate was cooled slowly to room temperature and then to 5°, and the edestin crystals were centrifuged and twice recrystallized from hot 5 % NaCl solution. The preparation was washed with distilled water until the wash liquors became slightly colloidal, and the paste was then dried in absolute alcohol, warm ether and finally in air. It was ground to a fine powder and sieved through muslin. In general it contained 10–12 % moisture giving on a water-free basis 0·1 % ash and 18·65 % N.

The *p*H of the freshly prepared protein suspended in CO_2-free water was usually between 5·3 and 5·5. The dried crystals, when dissolved in unbuffered NaCl give a sol containing only a minute amount of edestan formed in the washing process, and imparting only a slight haze to the solution.

(2) *Kinetics of edestan formation*

When increasing amounts of neutral salt are added to a native seed globulin at a *p*H value within the isoelectric zone, a concentration is reached at which the protein is freely soluble. If the native protein undergoes a change whereby, under the same conditions of *p*H and salt concentration, it is no longer soluble, it is said to be denatured. The solvent parameters will vary from protein to protein, some proteins being very soluble at low

temperatures and low ionic strengths, and others, like edestin, being soluble only when the salt concentration is fairly high. Native edestin is freely soluble in 1·71 *M* NaCl at 20°, and this solvent is a suitable one for separation of the undenatured and denatured components, provided that the protein is isoelectric or slightly on the alkaline side of the isoelectric zone. The isoelectric zone of edestin, from the results of several workers, is near *p*H 5·5. Michaelis & Mendelssohn [1914] found for example that the point of maximal precipitation in acetate buffers was at *p*H 5·6, and Svedberg & Stamm [1929] at *p*H 5·5. The osmotic pressure minimum according to Hitchcock [1921–22] lies between *p*H 5 and 6 and the isoelectric point as determined by cataphoresis between 5·5 and 6. The edestin used in the present experiments on suspension in CO_2-free water gave a *p*H value near 5·5. On acidifying with HCl in absence of salt it passed into clear solution at an equilibrium *p*H of 4·9, and when the original *p*H of such a solution was once more restored, it was found, after addition of neutral salt, that a portion of the protein remained undissolved. This portion, the edestan, is extremely insoluble in neutral salt over a wide *p*H range [Osborne, 1902, 1]. To measure therefore the proportion of edestin and edestan in a given system it is not so essential to define an exact *p*H at which the denatured portion is completely precipitated, as, for example, in the denaturation of albumins. It is more essential to ensure that the undenatured portion is completely dissolved in a suitable concentration of neutral salt. The amount of protein remaining undissolved under these conditions, or the amount of protein passing into solution, can give a measure of the extent of denaturation or degradation only if the production of edestan is unaccompanied by the formation of any considerable amount of salt-soluble breakdown products. This possibility has been ruled out by determining the homogeneity and sedimentation constants of the salt-soluble portion separated from the edestin-edestan system. Moreover, the assumption has been made, that over the *p*H range in which it was possible to measure the rate of edestan formation (between *p*H 3·9 and 4·9) the same type of denaturation change occurs.

*Edestan formation as a function of time, p*H*, protein concentration and temperature.* The dry protein was suspended in water at constant temperature, and after 3–4 min. was brought into solution by adding with rapid stirring a measured volume of *N*/10 HCl over a period of 5 sec. (The equilibrium *p*H of this solution was measured some 4 hr. later by means of the hydrogen-calomel electrode.) Aliquots of the solution were pipetted into a series of dry standard flasks containing solid NaCl, and at definite time intervals after acidification, solid NaCl was added to precipitate the protein, followed immediately by an amount of NaOH equivalent to the HCl initially added. The solutions in each flask were diluted to volume, the final NaCl concentration being 10 g./100 ml. of solution. The flasks were then equilibrated with frequent shaking for 2–3 hr. at 20°. The precipitated edestan was separated by filtration and the amount of salt-soluble protein was found by determining the N-content in an aliquot of filtrate using the Kjeldahl method. The amount of edestan present was thus obtained by subtracting the total salt-soluble protein from that originally present in the acid edestin sol.

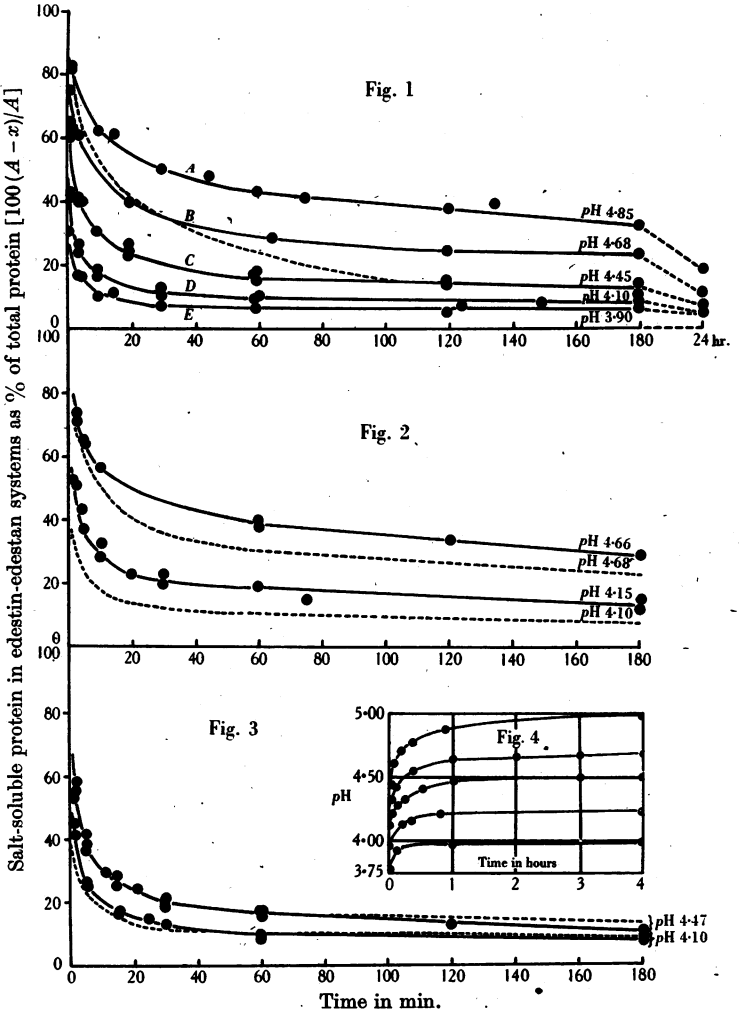
To check the reliability of the method in relation to the amounts of edestin and edestan in a given system, an edestin-HCl sol was prepared containing 0·986 g. of protein/100 ml., equilibrium *p*H 4·5, temperature 18°. 20 ml. aliquots were withdrawn at arbitrary intervals, 2·5 g. of NaCl and the necessary amount of NaOH were added and the solutions adjusted to 25 ml. After equilibration, the solutions were filtered and the N estimated in the filtrate. In an exactly parallel series, 50 ml. aliquots were treated in the same way and at the same times, but instead of estimating soluble N, the edestan was filtered off in weighed Gooch crucibles. It was washed with 20 ml. of 10 % NaCl, and successively with water, alcohol and ether. After drying to constant weight at 105° it was weighed. The added amounts of edestin and edestan thus determined approximate closely to the total protein concentration as determined directly by the Kjeldahl method on the protein acid sol (Table 1).

Table 1. *Edestin and edestan present at arbitrary time intervals as percentage of the total protein*

Edestin	Edestan	Total	Edestin	Edestan	Total
52·3	50·5	102·8	77·3	22·4	99·7
61·7	39·6	101·3	81·1	18·9	100·0
68·2	34·6	102·8	90·7	11·8	102·5

Mean 101·5

Figs. 1–3 represent graphically the amount of undenatured protein remaining after an acid edestan sol has been kept at various values of equilibrium *p*H, temperature and



Figs. 1, 2, 3. Salt-soluble protein in edestin-edestan systems for various values of equilibrium *p*H, plotted against time. Fig. 1: total protein concentration 1 %, temperature 20°. Fig. 2: total protein concentration 0·3 %, temperature 20° (full-line curves). Fig. 3: total protein concentration 1 %, temperature 2° (full-line curves). Fig. 4. *p*H of edestin-edestan systems as a function of time, protein concentration 1 %, temperature 20°.

protein concentration. The ordinates represent the value $100\,(A-x)/A$ (where A is the total protein concentration of the acid edestin sol, and x the amount of edestan at any

time t) and the abscissae the time in minutes. Each curve contains points derived always from two, and sometimes from three separate experiments.

In Fig. 1 only the equilibrium pH is varied, whilst the protein concentration remains at 1 % and the temperature at 20°. At all pH values there is a rapid initial production of edestan, followed by a much slower reaction in which the amount of edestan increases only slowly over a long period of time (24–48 hr.). At the equilibrium pH of 4·85 (curve A), the point at which edestin suspensions pass completely into solution, the formation of edestan is relatively slow, but below pH 3·9 (curve E) is too fast to be measured accurately.

In Fig. 2 the protein concentration is reduced to about 0·3 %, the temperature remaining at 20°, and the pH varied. The curves are contrasted with those in Fig. 1 (dotted lines) obtained at similar pH values with a higher protein concentration. It is seen that a decrease in protein concentration decreases the rate of edestan formation.

In Fig. 3 the temperature at which edestan formation is allowed to proceed is lowered to 2°, the protein concentration remaining at about 1 %. Again the curves for two different equilibrium pH values are compared with two from Fig. 1 (dotted lines) obtained at the same pH values but at a higher temperature. Within experimental error, the rate of edestan formation at 2° is the same as that at 20°. Even without reference to the complicating factors discussed below, this result is not surprising. Steinhardt [1937] has pointed out that the temperature effect in acid denaturation is often smaller than in alkaline solutions. The reason for this difference is that the denaturation mechanism must ultimately be related to the charge distribution of the molecule and therefore to the dissociation of carboxyl groups in this pH range. The effect of temperature on the dissociation is extremely small.

None of the curves in Figs. 1, 2 or 3 is unimolecular. The experimental methods and procedure have been varied without modifying essentially the course of the curve. In some experiments, for example, the amount of edestan formed has been measured in buffered salt solutions in order to eliminate any discrepancy arising from pH variations in solutions in which the protein itself was the sole buffering medium.

The apparent reaction kinetics of these curves were worked out in great detail, and it was found that they could be described in two ways: (1) by an equation representing a reaction of a high order.

$$Kt=\left(\frac{1}{(\alpha-1)\,A^{\alpha-1}}\right)\left(\frac{1}{(1-x/A)^{\alpha-1}}-1\right),$$

where A and x have their previous significance, and α is the order of the reaction; and (2) by the existence of two or more simultaneous unimolecular reactions. Because of the arbitrary criteria adopted for the study of denaturation rate, and the possible inhomogeneity of edestin, some deviation from the unimolecular law was to be expected, but it was felt that some specific explanation of such profound deviation must also be offered. An interpretation was sought in two ways. First, if edestin consists of several components, denaturing at different rates at any one pH value, then the undenatured protein recovered during the later part of the denaturation reaction, should, when exposed again to the same conditions of pH and temperature, denature at a rate different from that of the original protein. This view was not supported by experiment. Secondly, if the pH of the system, immediately after the addition of acid, migrates towards a more alkaline value, then the undenatured protein existing in the system at any time t will denature increasingly slowly as t progresses. The pH of the edestin-edestan systems were therefore measured with a sensitive glass electrode. This was immersed in a 1 % edestin-water suspension at room temperature, and $N/10$ HCl was then added with rapid stirring, and the pH recorded at intervals. It was then found that the initial pH at zero time might change to a value 0·25–0·6 units higher before an equilibrium pH was established.

The most important observation, however, was that the rate of *p*H change up to the equilibrium value was commensurate with the denaturation rate. This is illustrated in Fig. 4, where the rate of *p*H change up to the steady equilibrium value may be compared with the denaturation rates in Fig. 1. In the top curve of Fig. 4, which illustrates the changing *p*H of an edestin suspension brought into solution by a minimal amount of acid, the alkaline shift proceeds over many hours and cannot possibly be interpreted merely as the attainment of ionic equilibrium. Either new titratable groups are released, or the process of disorientation and depolymerization during denaturation causes a change in the magnitude of the pK values of groups such that they become titratable in this *p*H range. The significance of this effect in the interpretation of protein titration curves cannot be overestimated.

It is possible, however, to derive an approximate curve for the course of denaturation at constant *p*H. From Fig. 4 can be ascertained values of t at which the curves A, B and C of Fig. 1 have the same *p*H value of 4·4. For each value of t can be found the denaturation rate (dx/dt) and the corresponding value of $(a-x)$. Log (dx/dt) plotted against log $(a-x)$ gives an almost linear relation from which a series of denaturation rates and corresponding values of $(a-x)$ are derived. A final plot of dt/dx against x enables values of x at time t to be ascertained. The derived curve, shown as a dotted line in Fig. 1, still deviates from the unimolecular law, and quite apart from the large errors involved in a derivation of this kind, it can be seen at once from Fig. 1 that a departure from a first order reaction is to be expected. For example, the denaturation rate of curve A when $(A-x)$ lies between 90 and 100, compared with curve C when $(A-x)$ is 20, is much greater than the ratio 100 : 20, yet at these two points the systems have comparable *p*H values.

The explanation of a reaction which slows up in such a marked manner cannot, with the data at present available, be specific, but several possibilities are worthy of short consideration.

(1) Superficially, the slowing up can be described as an equilibrium between native and denatured protein, and although an explanation of this kind is common enough in denaturation studies, it does not imply a specific mechanism. We can, however, visualize that the interacting effects of the native molecule with the smaller depolymerized units, probably possessing new titratable groups and having a new configuration, are rather different from those between the molecules of the native protein. We might thus regard the native protein as protected by the denatured, in much the same way that a protein isolated from a complex mixture of other proteins is often less stable, other conditions being equal, than in the unpurified state.

(2) Denaturation of edestin involves extensive depolymerization of the molecule (cf. later sections) and such a process could be visualized as a series of step-wise reactions into multiples of the final fragmentary molecule of mol. wt. 17,000. The fact that the average size of the molecule in urea is 1/6, and in acid 1/18, that of the native molecule supports this hypothesis. At a given point in this chain of reactions, fragmentation is carried so far that some components cannot re-establish the soluble native configuration when the *p*H of the system is restored to a value within the isoelectric zone. By using loss of solubility as the criterion of denaturation we may thus be measuring only one step in a series of linked (exponential) reactions, the final rate being governed by the rate of formation of some intermediate product.

(3) The breakdown into the denatured component may proceed by several paths, and the rate of formation of denatured material is governed by the rates of several independent and simultaneous exponential reactions. The derived curve of Fig. 1 could thus be represented by an equation (*a*) involving four such reactions:

Undenatured component:

$$(a-x)=29{\cdot}7\ (0{\cdot}6913)^t+11{\cdot}4\ (0{\cdot}9053)^t+46\ (0{\cdot}9754)^t+12{\cdot}9\ (0{\cdot}9995)^t. \qquad (a)$$

The amount of denatured material at any time t calculated from this equation may be compared with the values obtained directly from the curve:

Time (min.)	0	5	10	15	20	30	40	60	90	120
$(A-x)$ calc.	100	65	54	47	42	35	30	23	17	15
$(A-x)$ from curve		65	54	47	42	35	30	23	17	15

The complexity of a protein molecule makes it probable that even within a homogeneous species of molecules the consecutive stages of denaturation do not proceed in an identical fashion in the case of every molecule, but the conception of simultaneous denaturation paths is even more likely if some proteins have the composition of isotropic systems [cf. Cohn, 1938].

Equation (*a*) can also be put in the symmetrical form

$$x=(-Cu_1e^{-\lambda_1 t}-Cu_2e^{-\lambda_2 t}-Cu_3e^{-\lambda_3 t}-Cu_4e^{-\lambda_4 t}+100^{-\lambda_5 t}),$$

which now resembles the equation for successive transformations as outlined in (2). In this particular case, the theoretical mathematical relations governing the values of the constants Cu_1-Cu_5 with the transformation constants $\lambda_1-\lambda_5$ (λ_5 being 0) do not hold, and if this explanation is at all valid, it would be necessary to invoke a greater number of transformations.

Such explanations cannot of course be offered with any sense of finality, but they serve to indicate that the problems connected with fragmentation and denaturation, difficult as they are, must be considered fundamental to an understanding of protein structure.

(3) *Ultracentrifuge studies*

A consideration of the kinetical data assembled in the previous section indicated that two main problems might be elucidated with the help of the ultracentrifuge: (1) whether edestin on the acid side of the isoelectric zone dissociates into smaller molecules of uniform size; and (2) whether the salt-soluble protein, obtained by adjusting the pH of the acid sol to a value within the isoelectric zone before the whole of the protein has transformed to edestan, is the native edestin of normal sedimentation constant. Concerning (1) certain data already exist. Adair & Adair [1934], from osmotic pressure data, have calculated that the mol. wt. of edestin in approx. $N/100$ HCl is 17,000. Moreover, Svedberg & Stamm [1929], in a study of the mol. wt. of edestin in buffered and unbuffered salt solutions, found that at pH 3·1 and 1·8 some breakdown into molecules of half the original particle weight occurred, and that some non-sedimenting material was also present. Between pH 5·5 and 9·7, on the other hand, the protein was monodisperse, and the mean sedimentation constant, converted into a basis of sedimentation in water, was $12{\cdot}8\times10^{-13}$ cm./sec. The diffusion constant of $5{\cdot}6\times10^{-7}$ cm.^{2}/sec. has since been revised by Polson [1937] to 3·93, thus necessitating a revision of the particle weight from 212,000 to 309,000. It must be emphasized that the pH stability of edestin as defined by Svedberg & Stamm is related to the breakdown of the molecule in presence of relatively high concentrations of salt. Under these conditions, the protein is precipitated almost quantitatively at the lower pH values, and the soluble molecules remaining in solution do not represent a change occurring in bulk, and may indeed be totally dissimilar from the product obtained when edestin chloride, ionizing in salt-free solution, is completely transformed to edestan.

The following experiments were carried out in collaboration with Mr J. St L. Philpot of Oxford, to whom I am indebted for ultracentrifuge runs and for their interpretation.

(*a*) *Edestan as a dissociation product of edestin.* A suspension of edestin in water was acidified to pH 4·5 with $N/10$ HCl and diluted to give a protein concentration of 1 %. The solution was kept at 20° for 24 hr. and the amount of protein then reconvertible into the salt-soluble form was 7 % of the total. The homogeneity of the protein chloride was surprisingly good, and the sedimentation constant was 2·63. Since the protein chloride

in such a solution is precipitated by a small amount of salt, we were interested to see if, at the point of incipient precipitation, we could produce a completely polydisperse system. When NaCl to a total concentration of 0·032 *M* was added to this solution, an opalescent sol was obtained, in which the homogeneity became moderate, the sedimentation constant rising to 3·0. A further increase in the salt molarity to 0·034 produced a milky fluid in which the homogeneity was bad, and which was found to contain coarse particles. Intermediate sizes between single molecules and aggregates were detectable, but only in low concentration and in such mobile equilibrium that they did not produce definite maxima.

The low sedimentation constants obtained in these experiments suggest a definite dissociation into smaller molecules; fibrous molecules, produced merely by molecular rearrangement and not by fragmentation, would reduce the sedimentation constant from 13 to about half its value. The exact calculation of molecular weights from runs carried out in salt-free solutions is rendered difficult, however, because of corrections due to a large electroviscous effect.

(*b*) *Monodispersity and sedimentation constant of salt-soluble protein recovered from an acid edestin sol.* A protein chloride sol prepared as described in (*a*) was kept for a short time after the addition of acid and was then neutralized to *p*H 5·5. NaCl was now added to 1·24 *M*, and after removal of edestan by filtration, the concentration of soluble protein was found to be 0·43 g./100 ml., representing a recovery of 44 % of the original protein. The homogeneity of the recovered protein was good, and the sedimentation constant 13·3 (corr.) agreed reasonably well with the value of 12·8 obtained by Svedberg and Stamm. Another portion of the same sol was diluted to a faint turbidity, and after cooling to 5°, gave the octahedra typical of native edestin.

From ultracentrifuge experiments, therefore, edestan is a depolymerized product of edestin, and under the conditions obtaining in our experiments, it is monodisperse. The salt-soluble protein recovered from a system in which edestin is being actively converted into edestan resembles the native protein both in crystalline form and in sedimentation constant. The presence of salt-soluble degradation products in sols of 'recovered' edestin was not observed.

(4) *X-ray diffraction patterns of edestin and edestan*

The diffraction patterns were obtained with the assistance of Dr W. T. Astbury and his colleagues in the Textile Physics Laboratory of Leeds University. Native edestin prepared and dried in the manner already described gives rise to a diffraction pattern displaying not only the characteristic backbone and side chain spacings of about $4\frac{1}{2}$ and 10 Å. respectively but an additional spacing of about 15 Å. Such a specimen was stored for 5 years in an air-tight bottle at room temperature without alteration in the spacings originally found. When dissolved in 5 % NaCl at *p*H 6·1, edestin may be precipitated quantitatively as the hydrochloride by adding *N*/10 HCl to *p*H 5·2. The diffraction pattern of this precipitate still retained the 15 Å. spacing of the crystalline protein, and could indeed be converted almost completely into the salt-soluble form by addition of alkali to *p*H 6·2. When, however, the hydrochloride was diluted with a large volume of water, it passed into solution, the *p*H falling to 4·6. After standing overnight, the *p*H was restored to 6·2, NaCl was added to 10 % strength and the insoluble edestan separated from the residual salt-soluble protein. The diffraction pattern of the edestan was now that of a denatured protein [Astbury & Lomax, 1935] and comparable with photographs of heat-denatured and urea-denatured protein.

This method of preparing edestan by initial separation from its salt solution with acid, and subsequent denaturation by allowing it to ionize, illustrates a constant source of danger in the preparation of proteins. Many proteins may be crystallized from concentrated salt solutions in the form of the protein sulphate or phosphate, which are relatively

stable as long as appreciable quantities of salt are present. If further fractionation is to be attempted, they must be dissolved in dilute buffer at a higher *p*H, and the salt concentration then increased to a point at which acidification is again possible without effecting denaturation. Alternatively, they may be dissolved directly in fairly strong salt solution. As an example, the albumin crystals obtained from skeletal muscle [Bailey, 1940] are quite stable in strong ammonium sulphate solution at *p*H 5·5. When separated from their mother liquor they dissolve on dilution, but after several minutes begin to precipitate as a denatured coagulum. Denaturation of this type is especially common in tissue extracts [Bailey, 1939] during the removal of salt by dialysis procedures.

(5) *Sulphydryl groups in edestan*

The appearance of SH groups often accompanies denaturation. Many proteins which do not give the nitroprusside reaction in the native state, do so after heat coagulation or after dissolving in denaturing solvents such as urea solutions [Hopkins, 1930] and solutions of guanidine salts [Greenstein, 1939]. The effect may be common both to proteins which exhibit depolymerization in these solvents, and to those which do not.

When edestin is dissolved in dilute HCl, SH groups can be detected immediately by the nitroprusside reaction. Experiments were designed to measure them in a quantitative fashion by reduction of the redox indicator phenolindo-2:6-dichlorophenol, according to the method of Todrick & Walker [1937]. Since there is a parallelism between the appearance of SH groups and reduction of the indicator, it is assumed that SH groups only take part in the reaction. Native edestin, for example, gives a negative nitroprusside reaction and decolorizes at *p*H 6·2 about 2·2 ml. of 0·05 % indicator per g. of protein, whilst freshly prepared edestan gives a positive SH test and decolorizes 20·6 ml. of indicator.

The experimental procedure was as follows. 2 g. of native edestin were suspended in 200 ml. of water and *N*/10 HCl was added to an equilibrium *p*H of 4·5. 20 ml. aliquots of the sol were transferred to a series of 8 Thunberg tubes, and after an appropriate time interval, 4 ml. of 3·3 *M* NaCl solution were added to each tube to precipitate the whole of the protein. Simultaneously the proportion of edestan present in the sol at the time of addition of salt was determined. When it became necessary to measure the reduction of indicator in systems containing only small amounts of edestan, it was not possible to complete the transference of aliquots to the Thunberg tubes in the short time interval between the point of addition of acid to the edestin suspension and the point where it was necessary to arrest edestan formation by addition of salt. In these cases, NaCl was added to the solution in bulk with rapid stirring, and aliquots of the suspended protein were transferred to the Thunberg tubes. The precipitated protein was centrifuged down in the tube itself, and after discarding the protein-free mother liquor, the precipitate was stirred with 2 ml. of *M*/5 phosphate buffer at *p*H 6·2, 2 ml. of 3·3 *M* NaCl, and an amount of 0·05 % indicator increasing by increments of 0·3 ml. along the tube series. The suspension was then diluted to 8·5 ml. exclusive of the volume of the protein paste. The tubes were evacuated several times, filled with N_2 after each evacuation and incubated at 38°. Under these conditions of *p*H and temperature, any undenatured edestin passes into solution. After 12–15 hr. the largest volume of indicator just decolorized by the indicator was recorded. A control experiment to assess the volume of indicator reduced by native edestin was also carried out.

In column 2 of Table 2 is tabulated the volume of indicator decolorized by 1 g. of a mixture of edestin and edestan. The reduction due to the latter alone is calculated by applying a small correction for the indicator reduced by edestin, and is given in column 3. Within the experimental error of this type of experiment, it is seen that the volume of indicator reduced by ¼ g. of edestan is constant. In other words, the number of SH groups present, after restoring the *p*H of an edestin chloride sol from 4·5 to a value in the isoelectric zone, is proportional to the concentration of the edestan component. The liberated

Table 2. *Reduction of phenolindo-2:6-dichlorophenol by edestin and edestan*

Amount of edestan in 1 g. total protein g.	Vol. of 0·05 % indicator reduced ml.	Vol. of 0·05 % indicator reduced/g. edestan ml.
0·0	2·2	—
0·17	5·4	21·2
0·18	5·6	21·2
0·52	12·9	22·8
0·87	15·6	17·6
0·87	17·8	20·1
	Mean	20·6

SH groups disappear on standing. If, for example, the formation of edestan is arrested 3 hr. after the addition of acid to the native protein, the volume of indicator decolorized by 1 g. of edestan is about 20 ml. and after 3–5 days only 4 ml.

If the same stoichiometrical relation holds for the amount of indicator reduced by free cysteine and by SH groups of the protein, the % SH in edestan, expressed as cysteine, is 0·34. This corresponds to about 1/4 of the total cystine as determined by colorimetric methods [Bailey, 1937, 1], a figure in good agreement with the SH groups liberated when edestin is dissolved in urea [Greenstein, 1939]. Since there is a tendency for free SH to condense with certain other hydrolysis products [Bailey, 1937, 1], some loss is incurred during the hydrolysis of a protein if part of the protein-S is present in this form. The present experiments indicate that some SH groups will in fact be liberated as soon as the acid necessary for hydrolysis is added to the protein.

(6) *Determination of total N, amide-N, tyrosine and tryptophan in edestin and edestan*

The liberation of SH groups is probably only one of several chemical changes brought about during denaturation. Osborne [1902, 1] first recorded that the N content of edestan (18·5 %) was slightly lower than that of edestin (18·69 %), but was cautious in attaching any great importance to the disparity between the two values. The high standard of accuracy maintained throughout Osborne's work suggests that the difference is greater than analytical error permits. It was decided to repeat Osborne's N determinations and to make in addition several other comparative determinations which have been found by experience to be accurate and reproducible. These were amide-N, tyrosine and tryptophan.

The edestin preparation was recrystallized three times, and the edestan was prepared by suspending it in water, adding $N/10$ HCl to pH 4·5, and after 24 hr. at room temperature, the acid was neutralized. NaCl was added to a concentration of 10 g./100 ml. and the insoluble protein was filtered off, washed first with warm saline, then with water until free from chloride, and finally dried in alcohol and ether.

Methods for the determination of total N, amide-N, and the Lugg colorimetric method [1937] for tyrosine and tryptophan have been discussed and utilized in a previous paper [Bailey, 1937, 2]. To gain the greatest possible accuracy in the colorimetric assays, the edestin and edestan hydrolysates were compared with each other, and the estimations were carried out on three separate hydrolyses, using aliquots in duplicate from each hydrolysis. The results are recorded in Table 3.

Table 3. *Analysis of edestin and edestan*

Total N %	Amide-N %	Tyrosine of edestan ÷ Tyrosine of edestin	Tryptophan of edestan ÷ Tryptophan of edestin
Edestin 18·6 ± 0·06	1·76	1·005 ± 0·005	0·97 ± 0·02
Edestan 18·4 ± 0·05	1·75		

Allowing for the maximal error in the total N determinations, there is a difference of at least 0·1 % in the two products. Whilst this work was in progress Hendrix & Dennis [1938]

have confirmed in a more exhaustive manner a similar N difference, obtaining 18·70 % for edestin and 18·50 % for edestan. Although the tyrosine content is unaltered, there appears to be a slight decrease in tryptophan, amounting to 2–3 % of the total. This loss was confirmed directly by investigating the dialysable non-protein N obtained from edestan solutions.

Dialysable non-protein N obtained from edestan. It is not unusual to find some nitrogenous material of low mol. wt. in protein crystals as for example in pepsin, where the non-protein N may be present in large amounts [Philpot, 1935; Steinhardt, 1938, 2]. If solutions of edestan are dialysed against several changes of distilled water, small amounts of nitrogenous material can be detected in the dialysate. 10 g. samples of edestin were suspended in 100 ml. of water, and HCl was added to an equilibrium *p*H of 4·5. The solution was placed in collodion bags and dialysed against frequent changes of distilled water over 2 days. The dialysates were combined and evaporated *in vacuo* at 35°, maintaining the *p*H at 5 by addition of dilute NaOH. In one experiment the edestan was precipitated by Stutze's copper reagent and the filtrate, without dialysis, was treated similarly. Here the yield of non-protein N was lower and the method was abandoned. As a control, an edestin suspension was dialysed in presence of 0·02 *M* Na_2SO_4, added to prevent the formation of colloidal solution. The dialysates were analysed for total and amino N (manometric Van Slyke) tryptophan and tyrosine, and in one instance for free ammonia.

Although the total N passing through the membrane is extremely small, it probably represents only a fraction of the total non-protein N, since the technique is not ideal for quantitative yields. From Table 4 it is seen that as much as 68 % of the total N in the dialysate may be accounted for as free ammonia and tryptophan, the latter being precipitated by the Lugg [1937] mercury reagent. Tyrosine is entirely absent. These results confirm the analytical data previously obtained, that whilst the tyrosine contents of edestin and edestan are identical, there is a slight decrease of tryptophan in the latter.

Table 4. *Dialysable N lost from 10 g. of edestan on dialysis*

Exp.	Total N in dialysate in mg.	Amino-N as % total N in dialysate	Tryptophan-N as % total N in dialysate	NH_3-N as % of total N in dialysate
(*a*)	1·33	43	23	—
(*b*)	2·0	37	19	—
(*c*)	0·9	41	33	35
(*d*)*	0·5	47	32	—
(*e*)†	0·25	—	Negative	—

* Precipitation by Stutze's reagent. † Control.

Discussion

We have observed that in the absence of salt edestan formation begins on the acid side of the isoelectric zone, at a *p*H value where only carboxyl groups are titrated, and we may infer that the acquisition of a small excess positive charge initiates the denaturation. The denaturation is affected, however, not only by the *p*H of the medium, but also by the nature of the anion of the added acid. For example, the chloride of the protein at 1 % strength is completely soluble at an equilibrium *p*H of about 4·9, whilst the sulphate and oxalate become soluble (and thus denature) at the lower *p*H values of 3·1 and 4·2 respectively. The insolubility of the protein salts of these latter acids accounts most probably for their greater stability. That definite compounds between edestin and added acid were possible was always stressed by Osborne, who characterized, in edestin preparations near the isoelectric zone, a mono- and di-chloride. The recent work of Steinhardt *et al.* [1941] adds most convincingly to the idea that a soluble protein such as egg albumin not only combines with H ions but possesses a specific affinity for anions.

Whilst edestin thus denatures on the acid side of the isoelectric point, it is clear that like many other seed globulins it is quite stable up to a *p*H of 9–9·5; Osborne indeed con-

cluded that seed proteins were less readily altered by alkalis than by acids. The real criterion, however, for determining the comparative stability of a protein towards acid and alkali is not the *p*H of the medium, but rather the amount of acid or of base bound at the point of incipient denaturation. In this respect it is apparent from the data of Hitchcock [1921–22] that edestin in the neutral range has only a slight base-binding capacity, and between *p*H 5 and 3·9, and between *p*H 5 and 9·8, combines with approximately equivalent amounts of acid or of base. The alkaline limit of the stability range is given by Svedberg at *p*H 9·8; certainly at *p*H 10·2 denaturation occurs. The protein thus appears to undergo denaturation when the excess negative or positive charge reaches a critical value of approximately the same order of magnitude. This is not true for all proteins, for in considerations of this kind, the charge distribution as well as the net charge must be taken into account.

Although the detailed mechanism of denaturation, as indicated in the kinetical section of the present paper, is obscure, the broad outlines may be inferred from all available evidence, adhering in a general way to the concepts of protein structure and denaturation which have been developed by Astbury and co-workers. Edestin itself has a large particle weight of 309,000, but on denaturation depolymerizes to 51,000 in concentrated urea solutions [Burk & Greenberg, 1930] and to 17,000 in dilute HCl [Adair & Adair, 1934]. These units are respectively about 1/6 and 1/18 the size of the native molecule. In the native state they possess a specific polypeptide pattern, and are integrated partly perhaps by some form of chemical linkage (e.g. S—S bonds), but chiefly by lateral attractions between neighbouring CO and NH groups and by interactions between free acid and basic groups of the side chains. The number of these latter groups is high, as can be seen from the following analytical data: glutamic acid, 19·2%; aspartic acid, 10·2% [Jones & Moeller, 1928]; arginine, 17·76% [Vickery, 1940]; lysine, 2·4%, histidine, 2·03% [Tristram, 1939]; amide-N, 1·73% [Bailey, 1937, 2]. Allowing for amidized COOH groups, they correspond to a total of 670 charged groups per molecule of 309,000. The spatial arrangement of such charges gives rise to a specific charge symmetry on which the stability of the molecule must ultimately depend, and this is capable of some variation, as reflected in a change of dipole moment, within definite limits of *p*H. Outside these limits, a further suppression in the ionization of acid or basic groups sets up within the molecule attractions and repulsions which, especially in the absence of small mobile ions, distort and finally destroy the unique polypeptide configuration.

Mirsky & Pauling [1936] have formulated the denaturation process in a somewhat similar fashion, but have used the conception of hydrogen bond formation as providing the means of holding the molecule together. Hydrogen bonds are formed between peptide nitrogen and oxygen, and between the amino and carboxyl side chain groups. In acid denaturation, protons are supplied to the electronegative portion of the H bond, and the polypeptide chain is then free to assume a new and denatured configuration. Astbury [1940], in a general review, has pointed out that only evidence of a very indirect kind has thus far been advanced for the existence of the H bonds in proteins.

The chemical changes which have been observed on denaturation may be correlated with the disorientation and fragmentation of the molecule. On depolymerization, we may expect a certain amount of hydrolytic cleavage, and the lower N content of edestan, inadequately accounted for by the loss of small quantities of ammonia-N and tryptophan, suggests that Osborne was correct in assuming edestan to be a hydrolytic product of edestin. This adding on of water suggests the liberation of new end-groups, and their presence has been surmised in an independent manner from the alkaline shift of *p*H which has been observed throughout the denaturation process. To account for the liberation of SH groups, one may suppose that they are produced either by the actual cleavage of the S—S groups during disorientation, or that they are present as such in the native molecule and become accessible to chemical reagents only when the molecule is disorganized. At

the present time, however, there is no really satisfactory theory to account for the appearance and disappearance of SH groups in the protein molecule. Whereas the SH groups detectable in native myosin may be increased, as in the case of many other proteins, by concentrated solutions of guanidine salts and urea, they disappear entirely in very dilute solutions of glycine and ammonium salts [Greenstein & Edsall, 1940]. Current views on the subject have been ably reviewed by Toennies [1940], but in addition a most promising line of enquiry has been opened up by Linderstrøm-Lang & Jacobsen [1941]. These authors find that the conditions under which the thiazoline ring of 2-methylthiazoline opens are similar to those in which SH groups appear in proteins. The folding of a cysteine-containing polypeptide chain to give a thiazoline grouping provides interesting possibilities for future research.

In the main, these chemical changes are symptomatic of a profound alteration in physical properties; something more, in fact, than the classical definition of denaturation affords. After breaking up into small monodisperse components, the protein precipitates in presence of salt over a very wide *p*H range and the asymmetric character of the degraded molecule becomes apparent from the visible appearance of the coagulum, which dries to a fibrous condition similar to that of myosin, a protein known to be extremely asymmetric. When once precipitated, the molecules adhere so firmly that re-solution in dilute HCl is not effected until the *p*H is relatively low, and even then a very viscous gel is produced. Although the process of depolymerization might in itself augment the solubility of the molecule, the tendency of richly charged disoriented chains to cohere at random is the factor most responsible for the loss of solubility. The comparison with myosin is again interesting. Here the native molecule is chain-like yet sufficiently hydrophilic to be solvated by neutral salts. In acid, the chains depolymerize lengthwise without any considerable change in the configuration of each individual portion, and after neutralization the protein still remains soluble in salts. But both native and degraded myosins lose their solubility most readily by any process of dehydration which assists lateral cohesion between the micelles. At this stage denatured myosin and edestan are very comparable from a structural point of view, and each may be orientated to a configuration resembling that of β-keratin [Astbury *et al.* 1935; Astbury & Dickinson, 1940]. In this connexion it is of especial interest to note that edestin was the first corpuscular protein to be transformed on denaturation into elastic threads and films which by X-ray interpretation are similar in structure to fibres such as hair and wool, thus providing the fundamental link between the structure of natural fibres and the typically corpuscular globulin [Astbury *et al.* 1935].

Summary

1. The formation of edestan by the action of HCl on edestin in salt-free solutions has been measured as a function of *p*H, protein concentration and temperature. The denaturation change, beginning on the acid side of the isoelectric zone, is relatively slow at an equilibrium *p*H of 5 and almost too rapid to measure below *p*H 4. The denaturation kinetics are characterized by a rapid initial reaction followed by a much slower one, and this deviation from the unimolecular law is due partly to a progressive shift of *p*H towards the alkaline side and partly to other factors which are briefly discussed. Since the rate of *p*H change is a function of the denaturation rate, it follows that edestan formation involves either the liberation of new titratable groups or a change in the *pK* of groups already present.

2. In the ultracentrifuge edestan is a monodisperse fragmentation product, the sedimentation constant, $s_{20} \times 10^{-13}$, being 2·6. The colloidal solutions formed by adding uniunivalent electrolyte to edestan solutions appear to involve a mobile equilibrium between the monodisperse component and very coarse particles. The salt-soluble portion recovered from systems in which edestan formation is actively proceeding is normal edestin of

sedimentation constant 13·6, and this is not accompanied by any large amount of salt-soluble degradation products.

3. The characteristic X-ray diffraction photograph of dry crystalline edestin is given also by the protein chloride when freshly precipitated from sodium chloride sols by addition of dilute HCl. When all salt is removed by washing, the protein chloride ionizes, denatures and gives after precipitation the photograph typical of other denatured proteins.

4. The number of SH groups appearing in the edestin-edestan system is proportional to the concentration of the denatured component, and represents about 1/4 of the total cystine-S.

5. Whilst the tyrosine content of edestan is unaltered, the total N and tryptophan contents are lower than those of edestin. Although small amounts of soluble N, largely NH_3-N and tryptophan, are liberated during the formation of edestan, the decrease in total N is due most probably to hydration of the molecule.

6. The mechanism of edestan formation is discussed.

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